The intercellular junctional complexes of retinal pigment epithelia

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Previous studies of the retinal pigment epithelium have suggested that its cells are interconnected by typical arrays of intercellular junctions. Using both conventional and freeze-fracture electron microscopy, we have found on the contrary that pigment epithelial cells in a wide variety of vertebrates (goldfish, frog, newt, mudpuppy, turtle, chicken, mouse, cat, and monkey) in fact have unusual junctional complexes. In all these species, each cell is completely girdled by a zonula occludens (tight junction) and a zonula adherens (intermediate junction). In addition, very large, macular gap junctions occur within the region occupied by the zonula occludens. The zonula adherens partially overlaps the zonula occludens, and thereby modifies the membrane cleavage properties of the latter junction. This yields a novel freeze-fracture pattern; the strands and grooves normally encountered on cleavage faces at zonulae occludentes are disrupted and fragmented in the region of the zonula adherens. Physiologic experiments demonstrate that, as might be expected from the presence of numerous gap junctions, pigment epithelial cells are electrically coupled. Our results confirm that pigment epithelial cells are joined by extensive zonulae occludentes (which form the "R" membrane) and zonulae adherentes. In addition, they show that gap junctions occur widely in pigment epithelia and that, as a result, pigment epithelial cells are functionally in electrical continuity with one another.

Key words: pigment epithelium, electrical coupling, junctional complex, intercellular junctions, gap junction, zonula adherens, zonula occludens.

The retina of the vertebrate eye consists of two portions: the neural retina, which transduces patterns of illumination into nerve impulses, and the pigmented retina, which plays an essential role in maintaining the neural component. The pigmented retina, or pigment epithelium, is a simple, cuboidal epithelium of homogeneous, polygonal cells whose apical microvilli interdigitate with the outer segments of receptor cells in the neural retina. By virtue of its pigmentation, it minimizes the intraocular reflection of light which has passed through the sensory retina without absorption. More importantly, the pigment epithelium helps to regulate the ionic environment of the sensory retina, stores the bleached chromophores of the visual pigments and par-
intercellular junctions occurring at the cells' epithelia with particular attention to their receptor cells' outer segments.5 We have studied the cells of pigment epithelia with particular attention to their junctional complexes, the assemblages of intercellular junctions occurring at the cells' apices. These junctions influence such general epithelial properties as transepithelial electrical resistance and ionic selectivity6 and intercellular communication.7, 8 An understanding of the junctions in this tissue is accordingly useful not only in the study of membrane contacts, but also in the investigation of retinal physiology.

Materials and methods

Histology. Pigment epithelia were obtained from healthy, adult animals, which included goldfish (Carassius auratus), frogs (Rana pipiens), newts (Notophthalmus [Triturus] viridescens viridescens), mudpuppies (Necturus maculosus), turtles (Pseudemys scripta elegans), chickens (Gallus gallus), mice (Mus musculus, strain C57BL/6J), cats (Felis domesticus), and monkeys (Ateles sp.). Eyes were dissected from dark-adapted animals anesthetized with either sodium thiopental (Pentothal, Abbott Laboratories, North Chicago, Ill.) for mammals, birds, and reptiles, or tricaine methane sulfonate (Finquel, Ayerst Laboratories, New York, N. Y.) for other species. After the anterior portions of eyes were removed, the neural retinae were carefully excised and the remaining eyecups fixed by immersion.

For transmission electron microscopy, eyecups were fixed in 3 per cent glutaraldehyde for 1 to 3 hours at 4° C. Buffering the fixative to pH 7.3 with either Sorensen's phosphate (0.12 M) for mammals, 0.08 M for other species) or sodium cacodylate (0.10 M and 0.08 M, respectively) gave similar results. After fixation, pigment epithelia were dissected from eyecups, cut into roughly 1 mm.2 pieces, and washed for 2 to 12 hours in the buffer system used in fixation; they were then postfixed for 2 to 4 hours at 4° C. in 1 per cent osmium tetroxide in the same buffer. In most cases (Figs. 1, 3, 7, 10, and 14), contrast was enhanced by adding 1.5 per cent potassium ferrocyanide to the osmium solution;6 otherwise, tissue was subsequently stained en bloc with 1 to 2 per cent uranyl acetate in sodium maleate buffer10 at pH 5.3. After dehydration in ethanol, tissue was embedded in Epon, cut on a diamond knife, stained with lead ion,11 and examined with a Philips EM 200 electron microscope operated at 60 kV.}

Material to be freeze-fractured was fixed by the procedure above, using Karnovsky's formaldehyde-glutaraldehyde fixative12 diluted to 67 per cent strength. Following fixation and dissection, pigment epithelia were equilibrated with 25 per cent glycerol in cacodylate buffer for 2 hours, frozen in liquid monochlorodifluoromethane at its freezing point, and stored on paper discs under liquid nitrogen. Specimens were freeze-fractured and shadowed with platinum on a Balzers Freeze Etch Machine. The replicas were supported by vacuum-evaporated carbon, then freed from tissue with chlorine bleach, rinsed in distilled water, and examined in a Siemens IA electron microscope at 80 kV.

Physiology. Eyes of N. maculosus or R. pipiens were removed from dark-adapted adult animals which had been either doubly pithed or anesthetized for 30 minutes in 0.1 per cent tricaine methanesulfonate. The anterior half of each eye was removed, the neural retina excised, and the remaining eyecup (with pigment epithelium uppermost) secured by pinning it to the soft plastic bottom of an experimental chamber. The preparation was maintained at 25° C. in a solution consisting of Leibovitz L-15 Medium (Grand Island Biological Company, Grand Island, N. Y.) diluted to 80 per cent strength with glass-distilled water, and supplemented with calcium chloride to a total Ca++ concentration of 4 mM. To prevent concentration of the solution by evaporation, the medium was totally exchanged at 30 minute intervals. Pigment epithelial cells showed stable resting potentials and uniform electrical coupling for at least 5 hours under these conditions.

Because the eyecup and pigment epithelium are opaque, the preparation was horizontally epi-illuminated with two microscope lamps equipped with heat filters. Individual pigment epithelial cells could be clearly discerned during experiments under a Zeiss WL microscope with a long-working-distance UD 16 objective lens (final magnification, about x100). Microelectrodes, which were mounted at an angle of roughly 45° to the vertical, reflected the horizontally incident light into the objective lens, and hence appeared bright against a darker background of epithelial cells. Electrode tips could be located to within about ± 5 μm under these circumstances; their separations were measured by a calibrated micrometer inserted in the microscope ocular.

Although most experiments involved pigment epithelial cells lying about halfway between the optic disc and the retinal margin, qualitatively similar results were obtained with cells from any part of the pigment epithelium, from the optic disc out to the ciliary margin.

Cells were penetrated, under microscopic observation, with glass microelectrodes filled by the fiberglass method13 with either 3 M. potassium
Figs. 1 and 2. Low power electron micrographs of junctional complexes between cells in the pigment epithelium of the frog. In both cases, the apices of the cells lie to the left.

Fig. 1. A conventional micrograph showing the three components of the junctional complex: a gap junction (GJ), membrane fusions of the zonula occludens (ZO, arrows), and the zonula adherens (ZA) with associated cytoplasmic filaments (F). SER, smooth endoplasmic reticulum; MB, myeloid body. x66,000.

Fig. 2. A comparable freeze-fracture preparation. The gap junctions (GJ) appears as clusters of particles on A cleavage faces (GJ-A), or as arrays of pits on corresponding B faces (GJ-B). The zonula occludens (ZO) is formed by numerous anastomosing grooves on the exposed B cleavage face (small arrows). The position of the zonula adherens (ZA) is evident from the displacement of the smooth endoplasmic reticulum (SER) from the adjacent cytoplasm. In this and all subsequent freeze-fracture preparations, the large arrow indicates the direction of the platinum shadow. x30,000.
chloride or 4 M. potassium acetate. The electrodes' d-c tip resistances were usually 50 to 100 MΩ; in some experiments, one electrode's tip was carefully broken or mechanically bevelled to lower its tip resistance to roughly 10 MΩ, and thus to facilitate the passage of current. Potentials were amplified with a high input impedance amplifier (Model M-4A, W-P Instruments, Hamden, Conn.), displayed on an oscilloscope, and photographed for later measurement. Each coupling measurement was repeated 2 to 5 times, and the uncertainty in the mean value estimated by the spread in measured potentials.

Results

Because its junctional complex is typical of those in all the species we have studied, we will first discuss the pigment epithelium of the frog (R. pipiens). The junctional complexes in this tissue occur roughly halfway down the sides of each cell, rather than at the cellular apices; nevertheless, they are structurally analogous to the terminal bars of other epithelia. Each junctional complex is divisible into three components (Figs. 1 and 2): gap junctions, which lie apically (vitread), a zonula adherens, which lies basally (sclerad), and a zonula occludens, which overlaps the other two junctions.

Gap junctions. Throughout the apical half of the junctional complex, transmission electron microscopy shows close apposition of the membranes of the adjacent cells (Fig. 1). At high magnification, however, it is apparent that, over much of this region of apposition, an extracellular space of 2 to 3 nm. separates the cells (Fig. 3). When colloidal lanthanum is added to fixative solutions, this space is filled by the dense tracer (Fig. 4). These observations suggest that the apical half of the junctional complex consists largely of gap junctions (nexuses). Freeze-fracture replicas confirm the presence of extensive gap junctions within the junctional complex. Aggregations of uniform, 9 nm. particles occur on A ("rough" or +") cleavage faces in the region of the junctional complex, while arrays of pits lie on B ("smooth" or -") faces (Figs. 2, 5, and 6). The gap junctions range in size from clusters of a few 9 nm. particles to maculae 2 μm in diameter containing hundreds of particles. The particles within a macula are not tightly packed in an hexagonal array, but characteristically cluster together in smaller groups, giving the macula a patchy appearance (Fig. 5). Gap junctions have been observed only in the region of the junctional complex and do not occur on more basal cell surfaces.

Zonula adherens. Beneath the gap junctions lies the most basal component of the junctional complex, the zonula adherens (Fig. 1). This prominent junction completely encircles each cell and extends for roughly 1 μm down each lateral cell border. The approximately 20 nm. extracellular space at the zonula adherens is only slightly denser than that elsewhere and shows no periodic substructure. The cytoplasm adjacent to the membranes, however, is highly specialized. Numerous 10 nm. diameter filaments, often in fascicles, run circumferentially around each cell at the level of the junction (Figs. 1 and 7). These filaments are largely confined to the cellular periphery, and do not form a terminal web. They are periodically embedded in dense, amorphous material which extends to the surface membranes. A few microtubules also run circumferentially about each cell, but lie nearer the cell's center than do the filaments. Other organelles, such as glycogen granules, endoplasmic reticulum, myeloid bodies, phagosomes, melanosomes, and mitochondria, are excluded from the cytoplasm in the region of the filamentous circumferential band. The absence of these organelles from adjacent cytoplasm clearly delineates the extent of the zonula adherens in freeze-fracture preparations (Fig. 2).

Zonula occludens. Transmission electron microscopy reveals that the extracellular space at the junctional complex is occasionally obliterated by focal fusions of the apposed membranes (Figs. 1 and 8). These fusions, which are indicative of zonulae oc-
Figs. 3 through 6. For legend, see opposite page.
Intercellular junctional complexes

Cludentes (tight junctions), occur both within the regions occupied by the gap junctions and within those occupied by the zonula adherens; they also occur apical to the gap junctions and between the gap junctions and the zonula adherens.

Freeze-fracture replicas of the frog's pigment epithelium show the sinuous features of the zonula occludens extending around the perimeter of each cell forming an unbroken belt (zonula) (Figs. 2 and 6). On A cleavage faces, the zonula occludens appears as 5 to 15 anastomosing ridges (Fig. 6); B cleavage faces show a complementary pattern of 5 to 15 grooves (Fig. 2). This figure correlates well with the number of focal membrane fusions observed by transmission electron microscopy.

In the region of overlap of the zonula occludens with the gap junctions, the particles of the gap junction lie on membrane cleavage faces among the anastomosing strands of the zonula occludens (Figs. 2, 5, and 6). Although gap junctions occur near the apical end of the junctional complex, we have never observed an instance in which a gap junction lay apical to all of the strands of the zonula occludens.

Transmission electron microscopy shows that the membrane fusions diagnostic of zonulae occludentes occur frequently in the region of the zonula adherens (Figs. 1 and 7). There is as yet no established correlate of the zonula adherens on membrane cleavage faces. Nevertheless, by the use of the cytoplasmic indications of the zonula adherens discussed above, we were able to use freeze-fracturing to demonstrate the overlap of the zonulae occludentes and adherentes. The basal portion of the zonula occludens, the portion overlapping the zonula adherens, regularly shows an unusual, roughened texture in freeze-fracture preparations (Figs. 6 and 9). This appearance seems to be due to the fragmentation of the zonula occludens' strands on A cleavage faces and the presence of particulate material in the grooves on B faces.

Interspecific comparison. The junctional complex of the frog's pigment epithelium is similar to that in each of the other species examined by transmission electron microscopy, including goldfish, newts, mudpuppies, turtles, chickens, mice, cats, and monkeys. The junctional complexes of pigment epithelia in species representative of the higher vertebrate classes are illustrated in Figs. 10 through 14. In each case, higher magnification micrographs show the junctional complex to include all three junctional types (gap junction, zonula adherens, and zonula occludens) in a geometric arrangement similar to that described for the frog.

Electrical coupling in pigment epithelia. The presence of gap junctions between pigment epithelial cells suggested that these cells might be electrically coupled (see 18 for references). We therefore undertook to demonstrate the flow of current from one cell to another. Because of the large size of its cells (roughly 30 μm in diameter and

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Fig. 3. A high magnification micrograph of a gap junction from the frog's pigment epithelium. In this conventionally fixed preparation, the 2 to 3 nm. "gap" is apparent (arrow). ×350,000.

Fig. 4. A gap junction similar to that shown in Fig. 3; the intercellular "gap" has been filled with dense tracer (arrow) after fixation in the presence of colloidal lanthanum. ×350,000.

Figs. 5 and 6. Freeze-fracture views of membrane cleavage faces at the junctional complex of frog pigment epithelial cells. The cells' apices lie to the tops of the figures.

Fig. 5. A large gap junction with both A and B cleavage faces exposed (GJ-A and GJ-B, respectively). This gap junction, as well as several smaller ones, is surrounded by strands of the zonula occludens. The clustering of the 9 nm. particles of the gap junction into smaller groups is evident on both fracture faces. ×47,000.

Fig. 6. A low power view showing numerous gap junctions of all sizes; the largest are indicated by GJ. The extensive network of strands of the zonula occludens (ZO) extends across the field. ×30,000.
Fig. 7. A tangential section at the level of the zonula adherens in the pigment epithelium of the frog. Each of the four cells in this field has a circumferential band of filaments (F) and microtubules (T) embedded in clumps of amorphous dense material. Two membrane fusions of the zonula occludens occur within the same region (arrows). Note that smooth endoplasmic reticulum (SER), mitochondria, and myeloid bodies (MB) are excluded from the cellular peripheries in the region of the zonula adherens. x27,000.

Fig. 8. A high magnification micrograph showing a membrane fusion at the zonula occludens of the frog’s pigment epithelium. x350,000.

Fig. 9. A freeze-fracture view of the zonula occludens in the pigment epithelium of the frog. The cells’ apices lie to the lower left of the field. The apical portion of the zonula occludens consists of relatively continuous strands on the exposed A cleavage face (A), and of smooth grooves on the B face (B). More basally, in the region of the zonula adherens, the strands on the A face are interrupted (A'), while the grooves on the B face appear partially filled with particulate material (B'). x55,000.
Figs. 10 through 14. Low power micrographs showing the junctional complexes of pigment epithelial cells in species representative of several vertebrate classes. In each instance, the cells' apices lie to the left. Each junctional complex includes a gap junction (GJ), a zonula adherens (ZA), and one or more membrane fusions of the zonula occludens (arrows).

Fig. 10. Goldfish (Pisces). Note the pattern of particles where the gap junction turns to give an en face view. ×80,000.

Fig. 11. Mudpuppy (Amphibia). ×95,000.

Fig. 12. Turtle (Reptilia). ×45,000.

Fig. 13. Chicken (Aves). ×35,000.

Fig. 14. Cat (Mammalia). ×75,000.
Fig. 15. Intercellular spread of current (electrical coupling) in the pigment epithelium of *Necturus*. As the upper part of the figure shows, a stationary microelectrode (*M*₁) is used to inject square current pulses (*I*) into one pigment epithelial cell. Another intracellular microelectrode (*M*₂) records the potential changes (*V*) produced by this current in another cell (here 19 cells distant, or about 510 μm away). The actual current and voltage records are shown. The lower portion of the figure shows the potential changes recorded in 10 cells at varying distances from the site of current injection. Error flags indicate the uncertainties in electrode positions and in the amplitudes of the measured potentials. The continuous curve plots the decrement of intracellular potential, as a function of distance, expected for a homogeneous epithelium of well-coupled cells (see text). There is excellent agreement of the experimental data with theoretic prediction.

20 μm in thickness), we chose to use the pigment epithelium of the mudpuppy (*N. maculosus*). A few successful experiments were also performed using frogs (*R. pipiens*). A microelectrode was used to pass square current pulses of 200 msec. duration into one pigment epithelial cell, while another electrode was placed in cells at varying distances from the first to measure transmembrane potentials due to the spread of current from cell to cell.

Pigment epithelial cells of *Necturus* gave good resting potentials (64.7 mV ± 3.1 mV, S.D., n = 43 cells penetrated), and showed clear-cut electrical coupling (Fig. 15). To rule out possible artifacts due to extracellular current flow above or below the pigment epithelium, each measurement was repeated with either the current passing or the recording electrode placed just over or beneath the cell in which a coupling potential had been recorded. Under these circumstances, passage of the same amount of current produced negligible potentials.

Similar results were obtained by using either hyperpolarizing or depolarizing currents; the current-voltage relationship was linear for currents of up to 50 nA. in either sense. No regenerative responses were observed in pigment epithelial cells.

The spatial decrement in the potential produced by current injected in one pigment epithelial cell closely follows the theoretic prediction for a uniform epithelial sheet with low-resistance coupling in all
The potential $V_x$ in a cell at distance $x$ from the source of injected current $I$ is given in this case by

$$V_x = c I K_0 \left( \frac{x}{\lambda} \right),$$

where $c$ is a constant of proportionality between the potential produced in a cell and the current injected, $K_0$ is the modified Bessel function of the second kind, order zero, and $\lambda$ is the space constant of the epithelium. The data for *Necturus* pigment epithelial cells (Fig. 15) fit this relationship well with $\lambda = 280 \mu\text{m}$ and $c = 0.08 \text{ mV/}\text{nA}$. Four other, less extensive measurements gave similar numerical results.

**Discussion**

These observations confirm previous morphologic investigations of pigment epithelia by verifying the presence of zonulae occludentes and adherentes in their junctional complexes. In addition, we have shown that these complexes generally include another specialized membrane contact, the gap junction, which has hitherto been noted in the pigment epithelium of only one species. Published micrographs of pigment epithelia of several other animals (rats, rabbits, sheep, and humans) resemble our Figs. 10 through 14; it seems likely that the junctional complexes in each of these cases includes gap junctions, as well as the previously noted zonulae occludentes and adherentes. The similarity of the junctional complexes in the pigment epithelia of species representing several phylogenetic classes suggests that this structure is of widespread occurrence among the higher vertebrates.

The junctional complexes of pigment epithelia differ in several respects from those found in other epithelia. First, desmosomes (maculae adherentes), which have been described in the junctional complexes of many other epithelia, are not found in the pigment epithelia of submammalian species, and are rare in the mammals. Second, the gap junctions of pigment epithelial cells are confined to the junctional complex, and have not been observed on more basal portions of the cell surface. It is our impression that the sizes of these gap junctions decrease with phylogenetic advancement; gap junctions are very frequently encountered and large in thin sections of fish pigment epithelia, smaller and fewer in those of amphibians and reptiles, and relatively rare in those of mammals.

Third, the zonula occludens overlaps the zonula adherens in the pigment epithelia of at least six of the species we have examined (goldfish, frogs, mudpuppies, newts, chickens, and mice). This arrangement, which has not been observed previously, leads to unusual membrane cleavage in freeze-fracture preparations. The strands (on A cleavage faces) and the grooves (on B faces) of the zonula occludens are of typical structure in the upper portion of the junctional complex. These features are disrupted in the lower portion of the complex where the zonula adherens overlaps the zonula occludens; the strands are interrupted, and the grooves partially filled with particulate material. The strands and grooves of the zonula occludens are normally complementary to one another; the grooves in B cleavage faces are evidently left when the material forming the strands is pulled away with the A face. This suggests that the unusual features observed in the lower portion of the zonula occludens result when much of the strand material remains with the B face during fracturing, forming a series of particles within the grooves. On the complementary A face, the missing material interrupts the strands of the zonula occludens. For the zonula adherens to form a mechanical attachment between cells, some material must connect the filaments within the cytoplasm of each cell with the adhesive material in the intercellular space. This would require local specialization of the cell surface membranes which could be responsible for the perturbation of membrane cleavage at the superimposed zonula occludens.

Freeze-fracture preparations confirm that the zonula occludens forms a broad, con-
tinuous belt around each cell. This supports evidence from thin sections that the pigment epithelium’s high transepithelial resistance (the \( R \) membrane\(^{20, 29-32} \)) and its role in the blood-retina barrier\(^{24, 33, 34} \) are due to the sealing of intercellular spaces by zonulae occludentes.

Gap junctions have been observed previously in the pigment epithelium of the mudpuppy and are common in the adjacent, embryologically related epithelia of the ciliary body and iris in mammals\(^{35} \) and in amphibians (unpublished observations). The present study documents another system in which gap junctions and electrical coupling both occur and thereby adds to the circumstantial evidence that the gap junction mediates coupling (see 18 for references). In addition, these observations contribute to the impression that epithelial cells in general are electrically coupled,\(^6 \) presumably by their ubiquitous gap junctions.\(^7 \) The electrical coupling of pigment epithelia is of particular interest because it contributes to a normal response to light. Electrical signals apparently spread from cell to cell within the pigment epithelium of the cat when the retina is locally illuminated\(^{16} \); it will be interesting to learn if these signals influence the metabolic contribution of the pigment epithelium to the visual process.

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